A novel non-invasive method to measure splenic filtration function in humans

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Supplementary Methods

Blood samples

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Regional Ethics Committee (n°3215 CPP Ile de France III). Blood samples were recovered from blood tubes drawn for medical care after written informed consent. Blood samples were collected on ethylenediaminetetraacetic acid (EDTA) from 36 patients with sickle cell anemia (SS and Sbeta° genotypes), from 3 splenectomized individuals and from 7 healthy donors (Etablissement Français du Sang).

Sample preparation

Blood samples were collected using EDTA tubes and centrifuged at 1500 rpm for 5 min. Ten µl of the RBC pellet were washed twice with 1 ml of buffer (PBS, 0.5% BSA) by centrifugation at 1500 rpm for 5 min at room temperature. RBCs were suspended in 1 ml of buffer, 80 µl of the suspension were added to 120 µl of buffer, and Hoechst 33342 (Life Technologies) is added at a final concentration of 40 µg/ml and incubated for 5 min at room temperature.

Imaging Flow Cytometry assay

Samples were acquired with the ImageStream®^{X} Mark II Imaging Flow Cytometer (IFC) (Merck Millipore) using the 405 laser. Analysis was performed using the IDEAS 6.2 software. The Hoechst-positive population was gated with reference to a negative tube (Figure 1A). To quantify the HJB-containing RBCs we developed a mask using the Modulation feature (a feature measuring the intensity range of an image normalized between 0 and 1) and the H Entropy feature (a texture feature used to determine if pixel values in an image follow a pattern or are randomly distributed). The above features were chosen due to

their ability to distinguish significantly the specific HJB-containing RBCs population from the non-specific staining. As shown in Supplementary Figure 1A, using the H Entropy Mean_M07_Hoechst on the x-axis and Modulation_Morphology (M07, Hoechst)_Hoechst on the y-axis, we set up the coordinates of the gate as: 3.356 - 5.9 on the X-axis and 0.38 - 0.456 on the Y-axis.

Flow Cytometry assay

Samples were prepared in the same manner as in the previous paragraph and acquired with a BD FACSCanto II Flow Cytometer using the 405 laser. 50,000 events were acquired for each sample using the DIVA software. Analysis was performed using the FCS Express 6 Flow Research Edition software (De Novo Software).

Blood smears and May-Grünwald-Giemsa staining

A drop of blood was placed on one end of a glass slide and dispersed over the slide's length using a coverslip. The blood smears were fixed with methanol for 30 sec at room temperature and stained with May-Grünwald-Giemsa staining (1/10 dilution in PBS) for 20 min. The slides were then washed with deionized water and left to dry. Photos were taken using an upright-microscope (Leica DM6000 B) with a 20x/0,4 HCX PL FLUOTAR, equipped with a DFC300 FX color camera. HJB-containing RBCs were quantified using the ImageJ software.

Splenic scintigraphy

The liver-spleen scintigraphy scan was performed using heat-denatured ^{99m}Tc RBCs as described by Adekile et al (Adekile AD, Owunwanne A, Al-Za'abi K, Haider MZ, Tuli M,

Al-Mohannadi S. Temporal sequence of splenic dysfunction in sickle cell disease. *Am J Hematol*. 2002;69(1):23-27).

Statistics

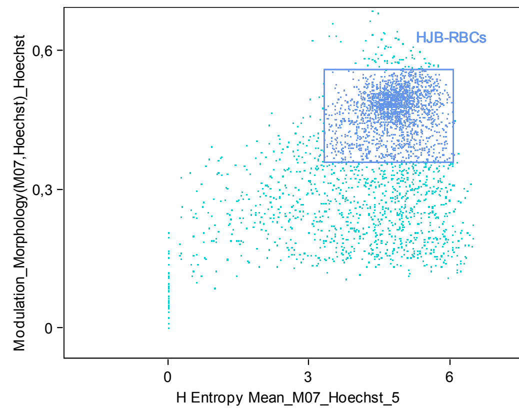
Data was analyzed by two-tailed Mann-Whitney or Wilcoxon test, Correlation (Pearson's correlation coefficient) and Linear Regression using the GraphPad Prism 7.00 software. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$ were considered significant.

Supplementary Table 1: %Hoechst-positive events and %HJB-RBCs in 10 splenectomized individuals (S), 7 healthy donors (C) and 36 SCD patients (P).

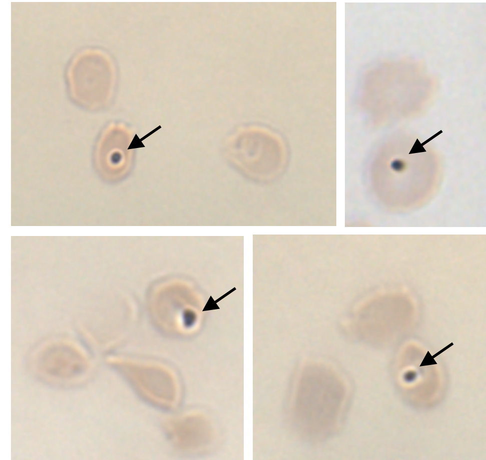
	Age (years)	%Hoechst-positive events	%HJB-RBCs
S1	66	13.00	9.80
S2	50	10.90	7.89
S3	34	10.90	9.43
S4	34	12.8	10.5
S5	22	14.2	11.6
S6	19	14.1	11.4
S7	11	7.8	6.2
S8	11	14.1	11.5
S9	9	12.8	10.2
S10	8	12.9	10.4
C1	45	1.30	0.30
C2	43	1.05	0.30
C3	38	1.20	0.60
C4	35	1.50	0.42
C5	31	1.10	0.30
C6	28	1.12	0.30
C7	27	1.30	0.00
P1	67	12.7	10.9
P2	61	13.4	10.59
P3	59	10.2	7.8
P4	59	18	15.8
P5	56	21.7	17.9
P6	53	9.3	7.85
P7	48	20.2	15.5
P8	40	21.6	17.5
P9	37	18.5	14.8
P10	36	14.1	11.5
P11	36	11.6	9.87
P12	1	3.2	2.24
P13	1	3.33	1.9
P14	1	0.51	0.32
P15	1	1.16	0.7
P16	1	0.29	0.06
P17	1	0.2	0.08
P18	1	0.09	0.07
P19	1	0.28	0.14
P20	1	0.08	0.02
P21	0.5	3.65	2.7
P22	0.5	1.3	0.6
P23	0.5	0.83	0.6
P24	0.5	0.82	0.61
P25	0.5	0.07	0.02
P26	0.5	0.09	0.02
P27	0.5	0.02	0.01
P28	0.5	0.2	0.03
P29	0.5	0.05	0.01
P30	0.5	3.14	2.3
P31	0.5	1.79	1.45
P32	0.5	0.33	0.23
P33	0.5	0.25	0.05
P34	0.5	1.23	0.71
P35	0.5	0.27	0.16
P36	0.5	0.2	0.11

Supplementary Figure 1

A



B



Supplementary Figure 1: (A) Dot plot representing the mask used to discriminate the HJB-RBCs from the non-specific staining using entropy mean feature on the x-axis and modulation morphology feature on the y-axis. (B) Images of HJB-RBCs colored by MGG staining taken from blood smears (magnification 20x).